available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Neuro-urology

Acetylcholine and Molecular Components of its Synthesis and Release Machinery in the Urothelium

Katrin S. Lips [a], *Julia Wunsch* [a], *Shirin Zarghooni* [a], *Thomas Bschleipfer* [b], *Konstantin Schukowski* [b], *Wolfgang Weidner* [b], *Ignaz Wessler* [c], *Ulrich Schwantes* [d], *Hermann Koepsell* [e], *Wolfgang Kummer* [a,*]

[a] *Institute for Anatomy and Cell Biology, Justus-Liebig-University, Giessen, Germany*
[b] *Clinic for Urology and Pedriatic Urology, Justus-Liebig-University, Giessen, Germany*
[c] *Department of Pathology, University of Mainz, Germany*
[d] *Dr. R. Pfleger Chemische Fabrik GmbH, Bamberg, Germany*
[e] *Institute for Anatomy and Cell Biology, Julius-Maximilians-University, Würzburg, Germany*

Article info

Article history:
Accepted October 16, 2006
Published online ahead of print on October 27, 2006

Keywords:
Aceytlcholine
Carnitine acetyltransferase
Choline acetyltransferase
Organic cation transporters
Vesicular acetylcholine transporter
Urothelium

Abstract

Objectives: Previous studies provided indirect evidence for urothelial synthesis and release of acetylcholine (ACh). We aimed to determine directly the ACh content in the urothelium and to characterize the molecular components of its synthesis and release machinery.

Methods: The study was performed on mouse bladder and abraded urothelium, and human mucosal bladder biopsies. ACh content was measured by high-performance liquid chromatography-electrochemical. Reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry served to investigate expression of ACh-synthesizing enzymes—choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT)—vesicular ACh transporter (VAChT), and polyspecific organic cation transporters (OCTs; isoforms 1–3). Transfected cells served to investigate whether the anticholinergic drug trospium chloride interferes with ACh-transporting OCTs.

Results: ACh is present in the urothelium in a nanomolar range per gram of wet weight. RT-PCR data support the presence of CarAT but not ChAT. VAChT, used by neurons to shuffle ACh into synaptic vesicles, is detected in subepithelial cholinergic nerve fibres, but not by RT-PCR or immunohistochemistry in the urothelium. OCT1 and OCT3 are expressed by the urothelium. The quarternary ammonium base trospium chloride inhibits cation transport by OCTs with a potency rank order of OCT2 (IC_{50} = 0.67 ± 0.42 μmol/l) > OCT1 (IC_{50} = 6.2 ± 2.1 μmol/l) > OCT3 (IC_{50} = 871 ± 177 μmol/l).

Conclusions: This study demonstrates a urothelial non-neuronal cholinergic system that differs widely from that of neurons with respect to molecular components of the ACh synthesis and release machinery. Consequently, these two systems might be differentially targeted by pharmacologic approaches.

* Corresponding author. Institute for Anatomy and Cell Biology, Justus-Liebig-University, Aulweg 123, 35385 Giessen, Germany. Tel. 49 641 9947000; Fax: +49 641 9947009.
E-mail address: wolfgang.kummer@anatomie.med.uni-giessen.de (W. Kummer).

1. Introduction

Synthesis and release of acteylcholine (ACh) is not restricted to specialized subsets of neurons but also occurs in a broad variety of non-neuronal cells, in particular in surface epithelia [1,2]. Recent studies suggest that this may apply for the bladder urothelium as well. Urothelial ACh has been proposed to be released into the bladder lumen to address nicotinic receptors on the luminal membrane of umbrella cells or to be released basally to act upon the detrusor and nerve fibres [3–6]. So far, however, there is only indirect experimental evidence for urothelial ACh. Yoshida et al. [6,7] measured ACh release from isolated human bladder strips and demonstrated a reduced release in preparations in which the epithelium had been removed. These findings are compatible with a urothelial release of ACh, but also with urothelial release of another factor that might trigger ACh release from deeper structures. In addition, immunolabelling of the urothelium with an antiserum directed against the ACh-synthesizing enzyme choline acetyltransferase (ChAT) has been reported [6,7], although these data still await validation by preabsorption experiments or other independent methods, such as Western blotting, determination of ChAT activity, or reverse transcriptase–polymerase chain reaction (RT-PCR).

On this background, we set out to determine the molecular components of a putative cholinergic system in the urothelium in more detail. First, ACh was detected in the urothelial cell layer by a high-performance liquid chromatography-electrochemical (HPLC-EC) method. Next, we employed RT-PCR and immunohistochemistry to investigate urothelial expression of (1) ChAT, the classical Ach-synthesizing enzyme in the nervous system and several non-neuronal cells; (2) carnitine acetyltransferase (CarAT), which is responsible for ACh synthesis in muscle cells [8]; (3) the vesicular ACh transporter (VAChT), which shuffles ACh from the cytoplasm into synaptic vesicle in cholinergic nerve terminals [9]; and (4) polyspecific organic cation transporters (OCTs; isoforms 1–3), which are able to translocate ACh directly across the plasma membrane [10]. In view of their polyspecific properties [11], we tested whether a commonly used anticholinergic drug, trospium chloride, interferes with ACh transport by OCTs. The study was performed on human and murine urothelium in parallel to evaluate whether the mouse may serve as a suitable model for experimental approaches addressing this system in the future.

2. Materials and methods

2.1. ACh assay

FVB mice were killed by inhalation of an overdose of isoflurane (Abbott, Wiesbaden, Germany). The bladder was carefully dissected, opened, and fixed in a Petri dish with the luminal surface facing upwards. A cotton-tipped applicator (Q-tip) was gently rubbed along the luminal surface and thereafter placed in 1 ml 15% formic acid in acetone (v/v). Two samples were taken from the luminal surface (about 2 cm^2) of human urinary bladders obtained from surgery. After standing on ice (30 min), specimens (material swiped off by the Q-tip) were frozen in liquid nitrogen. The bladder was then fixed in buffered 4% paraformaldehyde and processed as described for immunohistochemistry; then serial cryosections were stained with hematoxylin-eosin and evaluated by light microscopy. Only those specimens were included in the further analysis in which the basal lamina was not disrupted.

ACh was measured by cationic exchange HPLC combined with bioreactors and electrochemical detection with a detection limit of 10 fmol ACh per injection (20 μl) as described in detail elsewhere [12].

2.2. Reverse transcriptase–polymerase chain reaction

Murine samples were obtained by scraping off the urothelium as described above, but specimen holders were then placed in lysis buffer (RLT-buffer; Qiagen, Hilden, Germany) instead of formic acid/acetone. Further sample processing was as described in detail earlier [10]. Primer sequences are provided in Table 1.

NS20Y cells—a cholinergic murine forebrain neuroblastoma cell line (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany)—were used as a positive control for expression of components of the murine cholinergic system.

Human bladder mucosal biopsies were obtained from six female patients aged between 57 and 76 yr (mean: 67). All patients underwent endoscopic surgery primarily to remove bladder cancer or to examine the bladder wall by biopsies for carcinoma in situ or interstitial cystitis. Four additional mucosal biopsies were taken out of the trigonum (four patients) or the bottom (two patients) of the bladder with the use of endoscopic forceps. This procedure was approved by the local ethics committee, and informed patients gave signed consent. Processing of these specimens was identical to that of murine specimens, except that human specific primers were used (Table 1).

Caco cells—a human colonic epithelial cell line (German Collection of Microorganisms and Cell Culture)—served as a positive control for human epithelial ChAT expression. Additional complementary DNAs generated from airways during a previous study [10] were also used as positive controls.

2.3. Immunohistochemistry

Mice were killed by isoflurane inhalation; then the bladder was rapidly dissected, embedded in optimal-cutting-temperature compound, and shock-frozen in melting isopentane. For ChAT

Table 1 – Primers used for RT-PCR

Primer*	Sequence	Product length (bp)	Species	Accession
VAChT		571	Mouse, rat	NM_021712, NM 031663
for	GTATCCCGAGGAGCCTGAG			
rev	CTGTGTCCACTAACGCGATG			
OCT1		186	Mouse	NM_009202
for	GTAAGCTCTGCCTCCTGGTG			
rev	GCTGTCGTTCTCCTGTAGCC			
OCT2		169	Mouse	NM_013667
for	TACCGGAGTCTCCAAGATGG			
rev	GACCAAGTCCAGGAACGAAG			
OCT3		160	Mouse	NM_011395
for	CAGATATGGCAGGCTCATCA			
rev	TCACGATCACGAAGCAAGTC			
$ChAT_1$		400	Mouse	NM_009891
for	GAGCAGTATCATGCCTGAGC			
rev	CTGCAGGGACTTGTCATACC			
$ChAT_2$		183	Mouse	NM_009891
for	CCTGCCAGTCAACTCTAGCC			
rev	TCAGGGCAGCCTCTCTGTAT			
$ChAT_3$		178	Mouse	NM_009891
for	GCCCACCTATGAGAGTGCAT			
rev	GTACTCAGTTTGGGCCTGGA			
$ChAT_4$		1947	Mouse	NM_009891
for	TGCCTATCCTGGAAAAGGTC			
rev	GGCTGCCTCGAACTACAGAG			
M-$ChAT_1$		276	Mouse	D12487
for	AGAGAGGTGTGGCTGGTTTG			
rev	GGACCTTTTCCAGGATAGGC			
M-$ChAT_2$		360	Mouse	[29]
for	AGAGAGGTGTGGCTGGTTGTCTTG			
rev	CCAGTGCCCCCACTGCAGCAAACCCTGGCC			
M-$ChAT_3$		300	Mouse	[29]
for	TTCGTCGGAGGCTCTGCTACAGAACC			
rev	CCAGTGCCCCCACTGCAGCAAACCCTGGCC			
N-$ChAT_1$		346	Mouse	D12488
for	CCAGGCTCTATCATCTGAGG			
rev	GGACCTTTTCCAGGATAGGC			
N-$ChAT_2$		78	Mouse	[29]
for	GGATCCAGGCTCTATCATCTGAGG			
rev	CCAGTGCCCCCACTGCAGCAAACCCTGGCC			
R-$ChAT_1$		273	Mouse	D12490
for	CTGATCTGTTCAGCCTGTCG			
rev	GGACCTTTTCCAGGATAGGC			
R-$ChAT_2$		184	Mouse	D12490
for	CTGCAAATCAGGACGCTCAG			
rev	GGACCTTTTCCAGGATAGGC			
R-$ChAT_3$		300	Mouse	[29]
for	CATAGGCTGATCTGTTCAGCCTGTC			
rev	CCAGTGCCCCCACTGCAGCAAACCCTGGCC			
VAChT		157	Human	U10554
for	TACCCTACGGAGAGCGAAGA			
rev	CTGTAGAGGCGAACATGACG			
$ChAT_1$		151	Human/mouse	NM-020549
for	ATCGCTGGTACGACAAGTCC			
rev	ATCAGCTTCCTGCTGCTCTG			
$ChAT_2$		215	Human	NM-020549
for	GCAGGAGAAGACAGCCAACT			
rev	AGTCAGTGGGAATGGAGTGG			
hOCT1		198	Human	NM_153187
for	GACGCCGAGAACCTTGGG			
rev	GGGTAGGCAAGTATGAGG			
hOCT2		302	Human	NM_003058
for	TCGTCCATCGTCACCGAGT			
rev	TATCTCCGCCCAACAAATTC			

Table 1 (*Continued*)

Primer*	Sequence	Product length (bp)	Species	Accession
hOCT3		216	Human	NM_021977
for	GGAGTTTCGCTCTGTTCAGG			
rev	GGAATGTGGACTGCCAAGTT			
GAPDH		299	Mouse/human	AF106860
for	CGTCTTCACCACCATGGAGA			
rev	CGGCCATCACGCCACAGCTT			

bp: base pair; ChAT: choline acetyltransferase; for: forward; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hOCT; human organic cation transporter; OCT: organic cation transporter; rev: reverse; VAChT: vesicular acetylcholine transporter.
* M-, N- and R-ChAT primers are designed to amplify products from the noncoding M-, N- and R-exon, respectively, of the ChAT gene. All other primers are designed to amplify products within the coding regions.

immunohistochemistry, bladders from six additional mice were filled via a cannula with buffered 4% paraformaldehyde and immersed in the same fixative for 2 h, before being rinsed several times in 0.1 mol/l phosphate buffer (PB), immersed overnight in the same buffer supplemented with 18% sucrose for cryoprotection, and shock-frozen. From each patient from whom a mucosal biopsy had been obtained for RT-PCR analysis, a second biopsy was shock-frozen as described to be processed for immunohistochemistry. Cryosections (10 μm) were fixed either in acetone for 10 min at −20 °C or in Zamboni fixative (2% paraformaldehyde, 15% saturated picric acid in 0.1 mol/l PB) for 20 min and then processed for routine indirect immunofluorescence as described elsewhere [10]. Antibodies are listed in Table 2. The sections were evaluated by epifluorescence microscopy (Axioplan 2 imaging; Zeiss, Jena, Germany) or with a confocal laser-scanning microscope (TCS SP2; Leica, Mannheim, Germany).

The specificity of the primary antibodies was validated by (1) omission of the primary antibody, (2) preabsorption with their corresponding antigen (Table 2) at a concentration of 40 μg/ml for 1 h at room temperature before use in immunofluorescence, and (3) evaluation of immunofluorescence in genetically OCT-deficient mice (OCT1/2 double-knockout mice) by using tissues collected during a previous study [10].

2.4. *Expression of OCTs in epithelial cells and transport measurements*

Human OCT1, OCT2, and OCT3 were stably expressed in Chinese hamster ovary (CHO) cells by methods described in

Table 2 – Characteristics of antisera

Antigen	Host species	Dilution	Fixative	Source/reference
Primary antibodies for use in murine tissues				
ChAT, synthetic peptide, aa 282–295 of rat "common ChAT" sequence	Rabbit	1:8000	PFA	[23]
VAChT	Goat	1:800	Acetone, PFA	Biotrend, Cologne, Germany
OCT1, synthetic 21 aa peptide, near C-terminus	Rabbit	1:20	Acetone	Alpha Diagnostics, San Antonio, USA
OCT2, synthetic 21 aa peptide	Rabbit	1:400	Acetone	Alpha Diagnostics
OCT3, synthetic 18 aa peptide	Rabbit	1:400	Acetone	Alpha Diagnostics
Primary antibodies for use in human tissues				
ChAT; synthetic peptide, aa 282–295 of rat "common ChAT" sequence	Rabbit	1:8000	Zamboni	[23]
VAChT	Goat	1:500	Zamboni	Biotrend, Cologne, Germany
OCT1, synthetic 21 aa peptide, near C-terminus	Rabbit	1:20	Acetone	Alpha Diagnostics
OCT2, synthetic peptide, aa 533–547 of human sequence	Rabbit	1:200	Acetone	[10,30]
OCT3, synthetic peptide, aa 297–313 of human sequence	Rabbit	1:500	Acetone	[10]

Antigen	Host species	Dilution	Conjugate	Source
Secondary antibodies				
Rabbit-IgG	Donkey	1:2000	Cy3	Chemicon, Hofheim, Germany
Goat-IgG	Mouse	1:400	FITC	Sigma-Aldrich, Taufkirchen, Germany

ChAT: choline acetyltransferase; IgG: immunoglobulin G; OCT: organic cation transporter; PFA: paraformaldehyde; VAChT: vesicular acetylcholine transporter.
For all primary antisera, the corresponding antigen for preabsorption was available from the same source as the antiserum. In addition, CarAT isolated from pigeon breast muscle was obtained from Sigma-Aldrich, Taufkirchen, Germany, and used for preabsorption experiments with the ChAT antiserum.

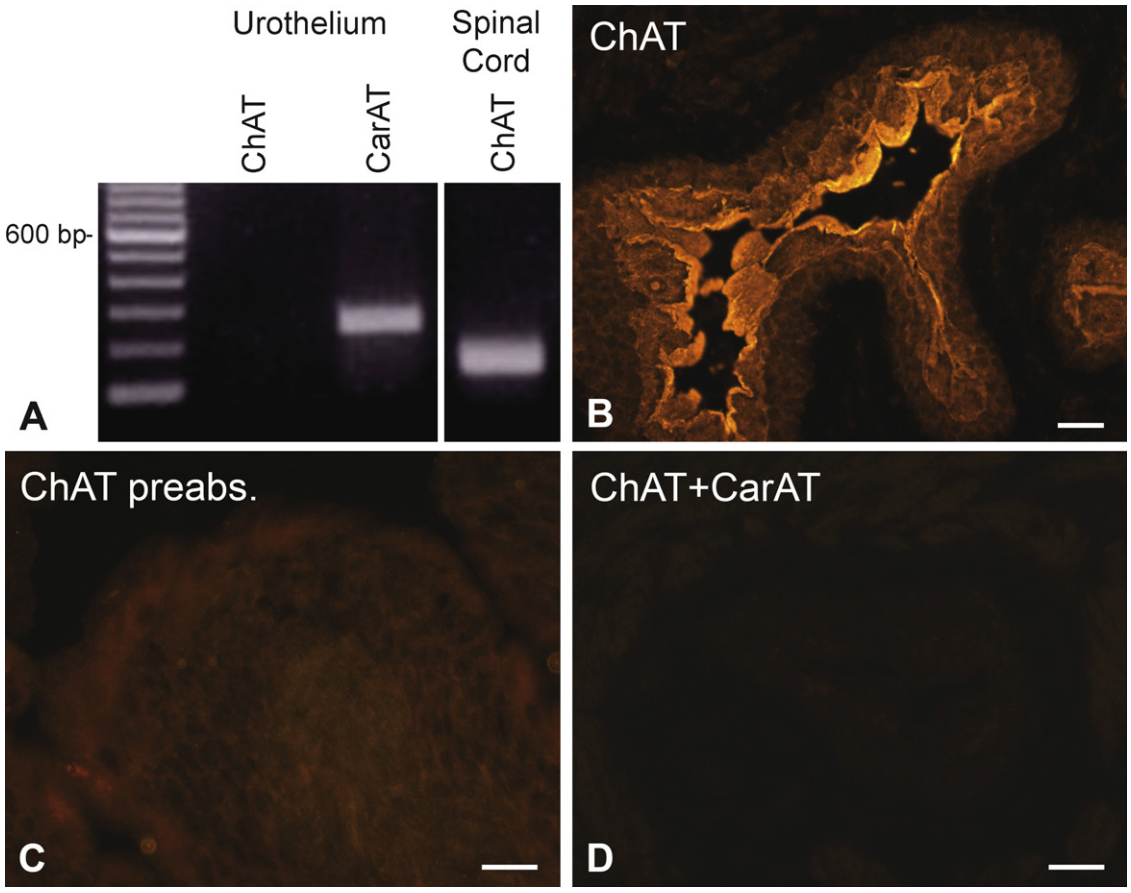

Fig. 1 – ChAT in murine urothelium. (A) Reverse transcriptase–polymerase chain reaction demonstrates the urothelial expression of CarAT, but not of ChAT (primer pair murine $ChAT_2$, Table 1). Spinal cord served as positive control for efficiency of detecting ChAT messenger RNA. (B–D) ChAT immunolabelling of the urothelium (B) can be successfully preabsorbed both with synthetic ChAT antigen (C) and with CarAT protein (D). Bar = 20 μm. bp: base pair. CarAT: carnitine acetyltransferase; ChAT: choline acetyltransferase.

detail earlier [10,13]. For transport measurements, confluent cells were washed with phosphate-buffered saline (PBS), suspended by shaking, collected by 10-min centrifugation at 1000 × *g*, and suspended at 37 °C in PBS. The cells were incubated for 1 s in PBS containing the prototypic substrate 0.2 μmol/l [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP), without inhibitor or in the presence of increasing concentrations of trospium chloride. Uptake was stopped by addition of ice-cold PBS containing 100 μmol/l quinine (stop solution), and the cells were washed three times with ice-cold stop solution. To measure uptake at 0-s incubation, ice-cold stop solution was added to the cells first, and radioactive substrates were added thereafter. Uptake rates were calculated from quadruplicate measurements after 0-s incubation and 1-s incubation. Uptake rates of [^{3}H]MPP in the presence of different concentrations of trospium chloride were calculated from measurements after 0 s and 1 s incubation. For each transporter, three or four independent experiments were performed in which four measurements were conducted for each concentration of trospium chloride. The Hill equation for multisite inhibition was fitted to individual (not shown) or to normalized and combined experiments (Fig. 8A–D). Mean values ± SD were calculated from the mean inhibitory concentration (IC_{50}) values of the individual experiments (Fig. 8D). The significance of differences between the mean IC_{50} values was estimated by one–way analysis of variance followed by Tukey comparison.

3. Results

3.1. *ACh and its synthesizing enzymes*

ACh concentration in the murine urothelium amounted to 0.22 ± 0.03 nmol/g wet weight (mean ± SEM; $n = 6$). Eight and 14 pmol, respectively, were measured in the two available samples of human urothelium. RT-PCR failed to detect ChAT messenger RNA (mRNA) both in murine (13 different primer sets tested) (Fig. 1A) and human (2 different primer sets tested) urothelium (Fig. 2A) despite positive results in human Caco cells (Fig. 2A), murine spinal cord (Fig. 1A), and murine tracheal epithelium (not shown). CarAT mRNA, however, was readily detectable (Fig. 1A). In immunohistochemistry, the

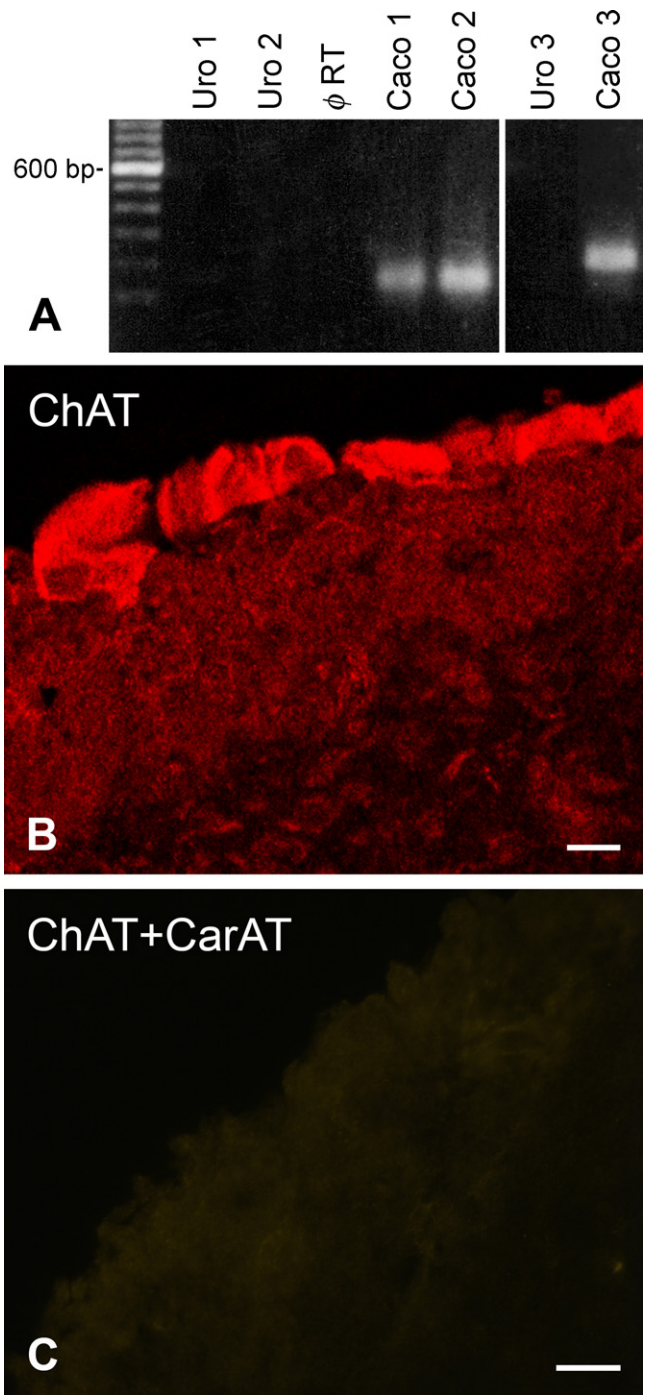

Fig. 2 – ChAT in human urothelium. (A) Reverse transcriptase–polymerase chain reaction failed to detect ChAT in the urothelium despite positive signals in Caco cells. (B, C) ChAT immunolabelling of the urothelium (B) can be successfully preabsorbed with CarAT protein (C). Bar = 20 μm. bp: base pair; CarAT: carnitine acetyltransferase; ChAT: choline acetyltransferase; Uro/CaCo 1+2: primer pair human $ChAT_1$; Uro/Caco 3: primer pair human $ChAT_2$ (Table 1).

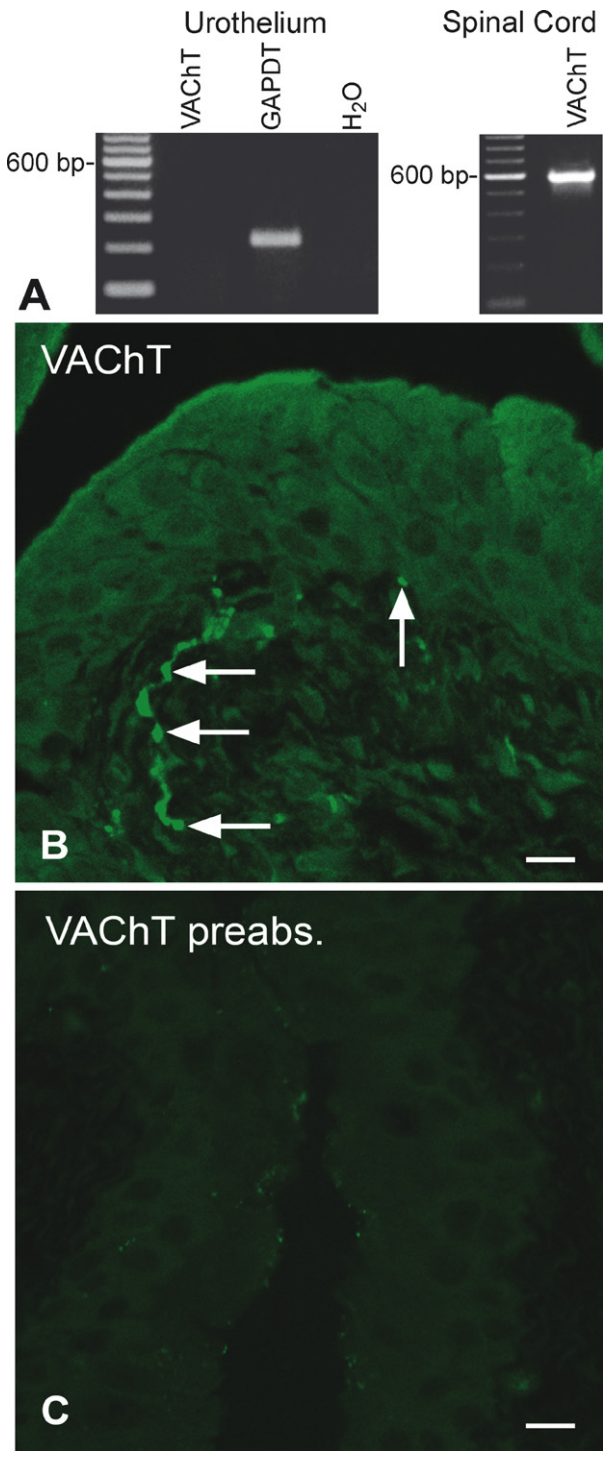

Fig. 3 – VAChT in murine bladder mucosa is undetectable by reverse transcriptase–polymerase chain reaction (A; spinal cord served as positive control). GADPH-specific primers confirmed efficacy of RNA isolation and reverse transcriptase. In immunohistochemistry, VAChT is restricted to subepithelial nerve fibres (arrows in B). (C) Preabsorption control. Bar = 20 μm. bp: base pair; GADPH: glyceraldehyde-3-phosphate dehydrogenase; H_2O: control run without template; VAChT: vesicular acetylcholine transporter.

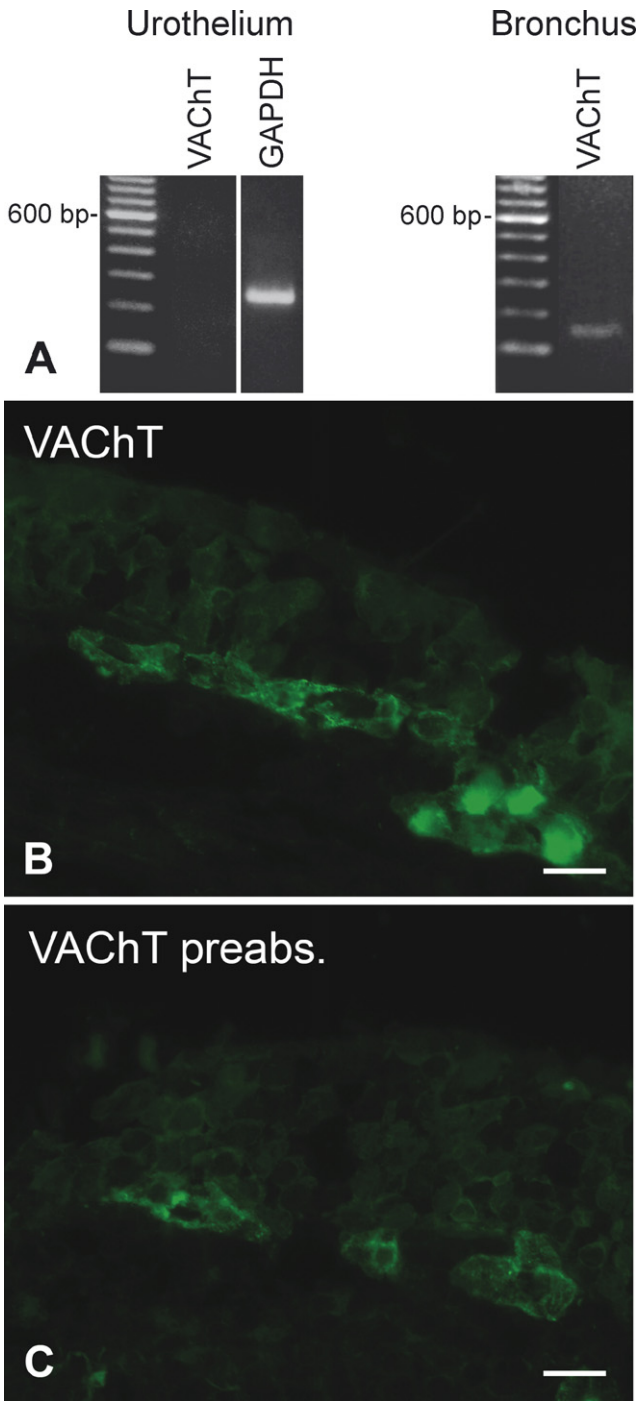

Fig. 4 – VAChT in human urothelium. Neither reverse transcriptase–polymerase chain reaction (RT-PCR) (A) nor immunohistochemistry (B) provides specific positive results. Nonspecific labelling of suburothelial structures persists after preabsorption of the antiserum (C). Human bronchial epithelium served as positive control for VAChT messenger RNA detection by RT-PCR, and GADPH-specific primers confirmed efficacy of RNA isolation and RT. Bar = 20 μm. bp: base pair; GADPH: glyceraldehyde-3-phosphate dehydrogenase; VAChT: vesicular acetylcholine transporter.

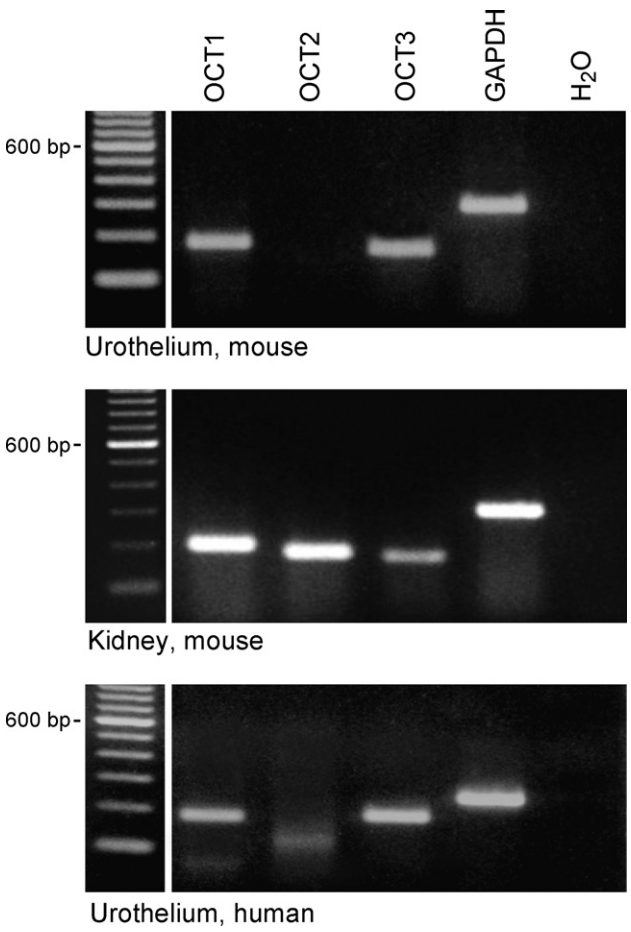

Fig. 5 – Expression of OCT isoforms, reverse transcriptase–polymerase chain reaction. OCT1 and 3 are expressed both in murine and human urothelium, whereas OCT2 is only faintly detected in human samples. Kidney served as positive control. bp: base pair; H_2O: control run without template; OCT: organic cation transporter.

ChAT antiserum distinctly labelled all cell layers in both murine and human urothelium (Figs. 1B–D and 2B). This labelling could be prevented by preabsorption of the antiserum with its corresponding synthetic antigen (Fig. 1C). In view of the RT-PCR data and the known structural similarity of ChAT with CarAT, we also preincubated the ChAT antiserum with CarAT isolated from pigeon breast muscle, which also resulted in loss of urothelial labelling (Figs. 1D and 2C). Hence, this immunolabelling was not suited to discriminate between ChAT and CarAT in the urothelium.

3.2. *Molecular components of the ACh release machinery*

VAChT, the vesicular transporter shuffling ACh from the cytosol into synaptic vesicles in cholinergic neurons, was detected neither by RT-PCR nor by immunohistochemistry in murine and

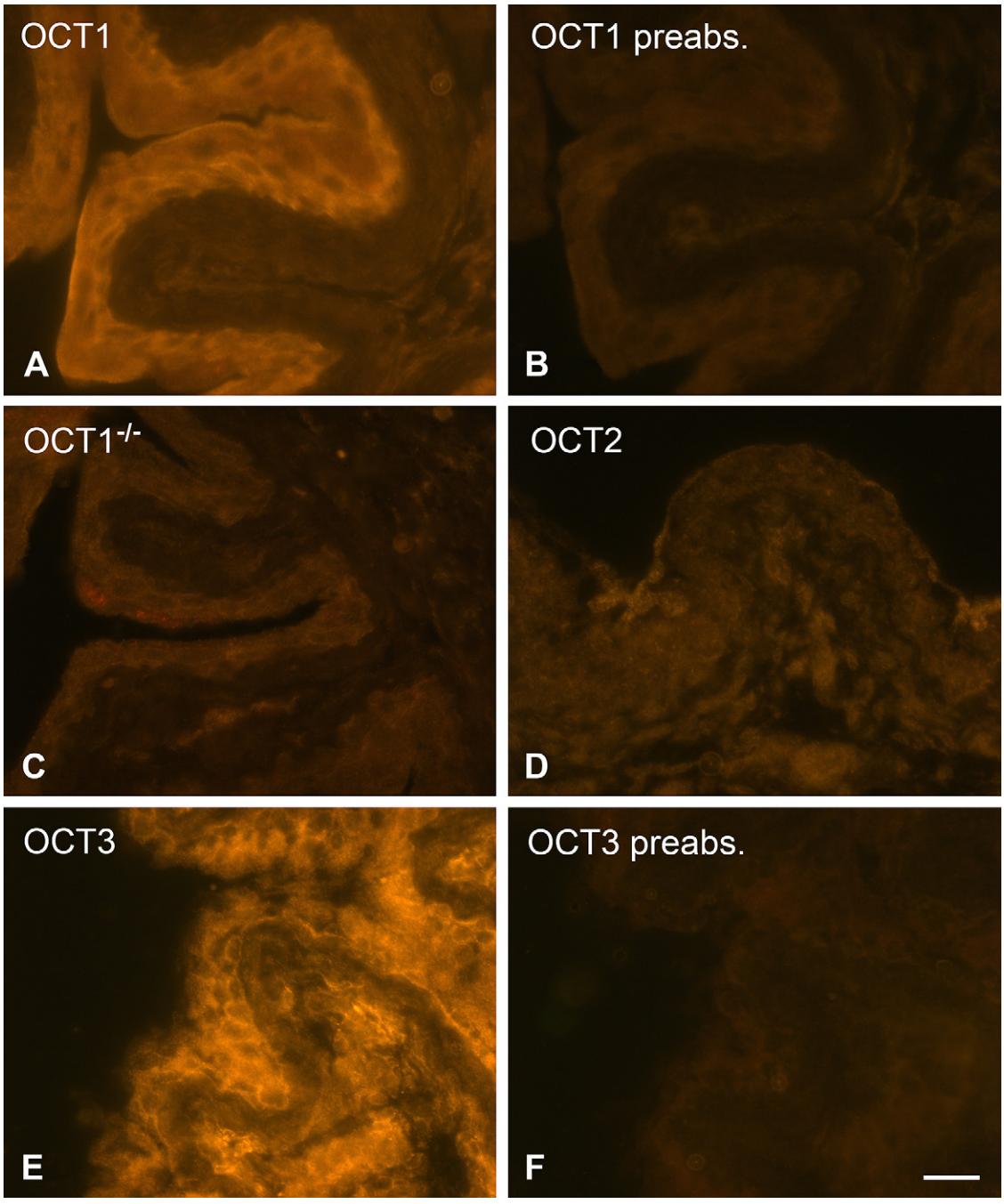

Fig. 6 – OCT immunohistochemistry, murine urothelium. OCT1 (A) and OCT3 (E) are primarily located in the basal and intermediate layer, OCT1 immunoreactivity is additionally seen in the apical membrane (A). Specificity is shown by preabsorption (B, F) and by the absence of OCT1 immunolabelling in the bladder section of an OCT1/2 double-knockout mouse (C). There is no specific OCT2 immunolabelling (D). Bar = 20 μm. OCT: organic cation transporter.

human urothelium (Figs. 3 and 4). Instead, numerous VAChT-immunoreactive nerve fibres were observed immediately underneath the urothelial basal membrane in the lamina propria (Fig. 3B). Among polyspecific OCTs, isoforms OCT1 and OCT3 were readily detected by RT-PCR both in murine and human urothelium (Fig. 5). Immunohistochemistry supported these data. OCT1 immunolabelling was observed throughout the epithelial layers with pronounced labelling of the intermediate and basal cells, and OCT3 immunoreactivity was nearly restricted to the basal cells (Figs. 6 and 7). Specific OCT2 immunolabelling was not obtained in the urothelium (Figs. 6 and 7).

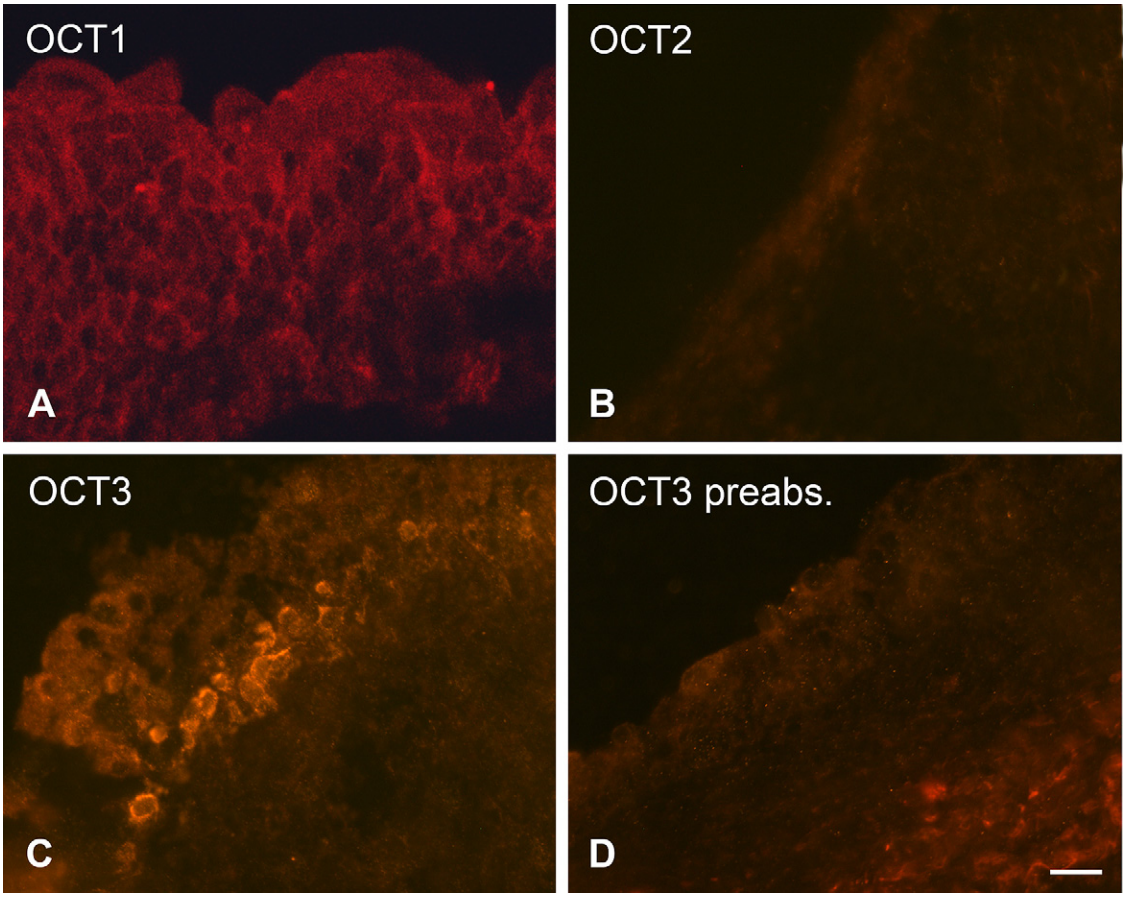

Fig. 7 – OCT immunohistochemistry, human urothelium. (A) OCT1 immunolabelling occurs throughout the epithelium with a slight predominance in the intermediate layer. (B) There is no specific OCT2 immunolabelling. (C, D) OCT3 is nearly restricted to the basal cells. Bar = 20 μm. OCT: organic cation transporter.

The ability of the anticholinergic drug trospium chloride to interfere with OCT-mediated transport was investigated in CHO cells stably transfected with either of the human OCT isoforms. Inhibition of MPP^+ uptake was taken as readout. The potency rank order of uptake inhibition by trospium chloride was OCT2 ($IC_{50} = 0.67 \pm 0.42$ μmol/l, $pIC_{50} = 6.17$ mol/l) > OCT1 ($IC_{50} = 6.2 \pm 2.1$ μmol/l, $pIC_{50} = 5.21$ mol/l) > OCT3 ($IC_{50} = 871 \pm 177$ μmol/l, $pIC_{50} = 3.06$ mol/l) (Fig. 8).

4. Discussion

This study unequivocally demonstrates the presence of ACh in the human and murine urothelium. The urothelial ACh tissue concentration is, in general, in the same size range as that of other surface epithelia [1]. In most epithelia, the Ach-synthesizing enzyme known from the nervous system, ChAT, has been identified at mRNA and protein levels and is likely to contribute the vast majority of ACh found in these tissues [1,14–16]. In this aspect, the urothelium differs markedly from these epithelia in that we were unable to detect ChAT mRNA by RT-PCR despite the use of several (13 in murine tissues, 2 in human tissues) primer pairs. One explanation may be the occurrence of a particular urothelial ChAT splice variant that might not be recognized by the primers used in our study. Indeed, a great diversity in ChAT transcripts is known, including variants in the noncoding and coding region [17–19]. However, there is no variant known so far that would not have been recognized by at least one of the primer pairs used in the present study. At first sight, the lack of detection of urothelial ChAT mRNA might contradict a previous report on ChAT immunolabelling of the human urothelium [6,7]. This finding, however, could also be reproduced in our hands with the use of another ChAT antiserum. On the basis of our negative RT-PCR results for ChAT, the presence of CarAT mRNA, the potential of CarAT to synthesize ACh [8], and the structural similarities of ChAT and CarAT, we considered the possibility that the positive

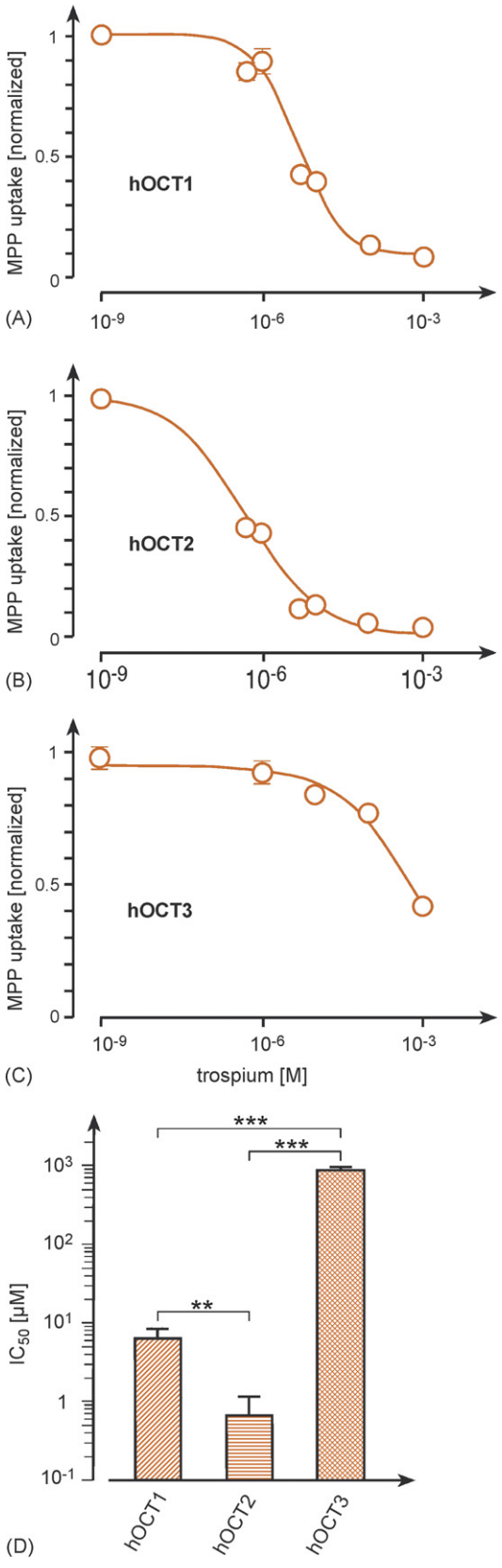

Fig. 8 – Inhibition of the human organic cation transporters OCT1, OCT2, and OCT3 by trospium chloride.
(A–C) Measurements in CHO cells that were stably transfected with human OCT1, OCT2, or OCT3. Uptake of 0.2 µmol/l [^{3}H]MPP was measured in the absence and presence of various concentrations of trospium chloride. The uptake of 0.2 µmol/l [^{3}H]MPP in nontransfected CHO cells or in CHO cells stably transfected with an empty vector was less than 5% compared with the uptake of CHO cells transfected with OCTs. Each data point in A–C represents a mean value ± SEM from 12–16 individual measurements. The Hill equation was fitted to the data. (D) Comparison of IC_{50} values for inhibition of [^{3}H]MPP by trospium chloride. Mean values ± SD that were calculated from IC_{50} values obtained by fitting the Hill equation to three or four individual experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. CHO: Chinese hamster ovary; IC_{50}: mean inhibitory concentration; [^{3}H]MPP: [^{3}H]1-methyl-4-phenylpyridinium; OCT: organic cation transporter.

immunolabelling obtained with the ChAT antiserum might be caused by CarAT. Indeed, CarAT antigen successfully preabsorbed ChAT immunolabelling of the urothelium. Thus, the data are in favour of CarAT being the major Ach-synthesizing enzyme in the urothelium, although the presence of a still unknown ChAT variant or of mRNA levels too low to reach detection limit in conventional RT-PCR cannot be excluded.

With respect to the molecular components of the ACh release machinery, the urothelium markedly differs from cholinergic nerve terminals in that VAChT, the transporter shuffling ACh from the cytoplasm into synaptic vesicles in nerve endings, was detected neither at mRNA nor protein level. In general, the functional role of VAChT outside the nervous system is unclear. VAChT mRNA and/or protein have been detected in some, but by far not all, non-neuronal cholinergic cells, including lymphocytes [20], endothelial cells [21,22], selected cell types of epithelia [10,16], and the placenta [23], but functional evidence for vesicular release of ACh from non-neuronal cells so far has not been obtained. In contrast, non-neuronal ACh release has clearly been demonstrated under conditions of pharmacologic inhibition of VAChT or of the vesicular exocytotic machinery in various tissues [24,25] including strips of the human bladder [6]. Instead, non-neuronal ACh release appears to occur directly from the cytoplasm across the plasma membrane. Using antisense oligonucleotides, Wessler et al. [26] obtained evidence for ACh release via OCT1 and OCT3 from human placenta villi. When expressed in *Xenopus* oocytes, human and rat OCT1 and OCT2, but not OCT3, efficiently transport ACh [10]. In the tracheal epithelium, ACh content is significantly elevated in OCT1/2 double-knockout mice indicating impaired release due to the lack of

these transporters [27]. Hence, it can be expected that OCTs, particularly OCT1, which we have demonstrated to occur in the human and murine urothelium, represent a major ACh release mechanism from the bladder mucosa as well. This finding does not exclude the simultaneous occurrence of additional ACh transporters because recent microdialysis studies on human skin revealed ACh release that was resistant to inhibition of both vesicular exocytosis and OCTs [28].

In view of the bidirectionality of OCTs, it also has to be considered that OCT1 residing in the urothelial plasma membrane mediates ACh uptake into the cell. Cation transport by OCTs is electrogenic, and direction of transport is determined by membrane potential and substrate concentration [11]. Direct evidence for concentration-dependent bidirectional transport of ACh has indeed been presented for OCT1-expressing *Xenopus* oocytes [10]. In view of the presence of cholinergic nerve terminals immediately underneath the urothelium, which we demonstrated by VAChT immunohistochemistry, it can be expected that a locally high ACh concentration can be reached upon stimulation of these nerve endings, and may lead to ACh loading of neighbouring epithelial cells via OCT1.

Because the molecular components of the urothelial ACh release machinery differ from those in the nervous system, the option arises to address these systems differentially by pharmacologic approaches. A characteristic feature of OCTs is their polyspecificity, and accordingly, many widely used drugs are also OCT substrates or interfere with OCT-mediated transport [11]. Here we show that this feature also applies to trospium chloride, an anticholinergic compound commonly used for treatment of overactive bladder. In view of the present data, it has to be considered that, in addition to the well-established muscarinic receptor antagonist properties, this and chemically similar compounds also may act as inhibitors of ACh release from the epithelium.

In conclusion, the present study demonstrates a urothelial non-neuronal cholinergic system that differs widely from that found in neurons with respect to the molecular components of the ACh synthesis and release machinery. Consequently, these two systems can be differentially targeted by pharmacologic approaches.

Conflicts of interest

This study was supported by the DFG, grant SFB 487/A4 to H.K. and Li 1051/1-1 to K.S.L., and by a project bound donation of the Dr. R. Pfleger Chemische Fabrik GmbH.

U. Schwantes is employed by Dr. R. Pfleger Chemische Fabrik GmbH, Bamberg, Germany.

Acknowledgements

The authors thank M. Bodenbenner for expert technical assistance and K. Michael for preparing the figures.

References

[1] Klapproth H, Reinheimer T, Metzen J, et al. Non-neuronal acetylcholine, a signalling molecule synthesized by surface cells of rat and man. Naunyn Schmiedebergs Arch Pharmacol 1997;355:515–23.

[2] Wessler I, Kirkpatrick J, Racke K. The cholinergic ''pitfall'': acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 1999;26:198–205.

[3] de Groat WC. The urothelium in overactive bladder: passive bystander or active participant? Urology 2004; 64:7–11.

[4] Birder LA. More than just a barrier: urothelium as a drug target for urinary bladder pain. Am J Physiol Renal Physiol 2005;289:F489–95.

[5] Beckel JM, Kana A, Lee SJ, de Groat WC, Birder LA. Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells. Am J Physiol Renal Physiol 2006;290:F103–10.

[6] Yoshida M, Inadome A, Maeda Y, et al. Non-neuronal cholinergic system in human bladder urothelium. Urology 2006;67:425–30.

[7] Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A. Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. Urology 2004;63(3 suppl 1):17–23.

[8] Tucek S. The synthesis and release of acetylcholine in normal and denervated rat diaphragms during incubations in vitro. J Physiol 1982;322:53–69.

[9] Eiden LE. The cholinergic gene locus. J Neurochem 1998;70:2227–40.

[10] Lips KS, Volk C, Schmitt BM, et al. Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol 2005;33:79–88.

[11] Koepsell H, Schmitt BM, Gorboulev V. Organic cation transporters. Rev Physiol Biochem Pharmacol 2003;150: 36–90.

[12] Reinheimer T, Bernedo P, Klapproth H, et al. Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. Am J Physiol 1996;270:L722–8.

[13] Busch AE, Karbach U, Miska D, et al. Human neurons express the polyspecific cation transporter hOCT2,

which translocates monoamine neurotransmitters, amantadine, and memantine. Mol Pharmacol 1998;54: 342–52.
[14] Grando SA, Kist DA, Qi M, Dahl MV. Human keratinocytes synthesize, secrete, and degrade acetylcholine. Invest Dermatol 1993;101:32–6.
[15] Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 1998;77:59–79.
[16] Proskocil BJ, Sekhon HS, Jia Y, et al. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 2004; 145:2498–506.
[17] Oda Y. Choline acetyltransferase: The structure, distribution and pathologic changes in the central nervous system. Pathol Int 1999;49:921–37.
[18] Tooyama I, Kimura H. A protein encoded by an alternative splice variant of choline acetyltransferase mRNA is localized preferentially in peripheral nerve cells and fibers. J Chem Neuroanat 2000;17:217–26.
[19] Robert I, Quirin-Stricker C. A novel untranslated ''exon H'' of the human choline acetyltransferase gene in placenta. J Neurochem 2001;79:9–16.
[20] Tayebati SK, El-Assouad D, Ricci A, Amenta F. Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 2002;132:147–55.
[21] Haberberger RV, Bodenbenner M, Kummer W. Expression of the cholinergic gene locus in pulmonary arterial endothelial cells. Histochem Cell Biol 2000;113:379–87.
[22] Kirkpatrick DJ, Bittinger F, Unger RE, Kriegsmann J, Kilbinger H, Wessler I. The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. Jpn J Pharmacol 2001;85:24–8.
[23] Pfeil U, Vollerthun R, Kummer W, Lips KS. Expression of the cholinergic gene locus in the rat placenta. Histochem Cell Biol 2004;122:121–30.
[24] Wessler I, Roth E, Schwarze S, et al. Release of non-neuronal acetylcholine from the human placenta: difference to neuronal acetylcholine. Naunyn Schmiederbergs Arch Pharmacol 2001;364:205–12.
[25] Moffat JD, Cocks TM, Page CP. Role of the epithelium and acetylcholine in mediating the contraction to 5-hydroxytryptamine in the mouse isolated trachea. Br J Pharmacol 2004;141:1159–66.
[26] Wessler I, Roth E, Deutsch C, et al. Release of non-neuronal acetylcholine from the isolated human placenta is mediated by organic cation transporters. Br J Pharmacol 2001;134:951–6.
[27] Kummer W, Wiegand S, Akinci S, et al. Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. Respir Res 2006;7:65.
[28] Schlereth T, Birklein F, Haack KA, et al. In vivo release of non-neuronal acetylcholine from the human skin as measured by dermal microdialysis: effect of botulinum toxin. Br J Pharmacol 2006;147:183–7.
[29] Misawa H, Ishii K, Deguchi T. Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region. J Biol Chem 1992;267:20392–9.
[30] Gorboulev V, Ulzheimer JC, Akhoundova A, et al. Cloning and characterization of two human polyspecific organic cation transporters. DNA Cell Biol 1997;16: 871–81.